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Anesthesiology (Hagerstown), (1996) Vol. 84, No. 5, pp. 1205-1214.

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Thank-you!

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Knockdown of PSD-95/SAP90 delays the development of neuropathic pain in rats

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Received 25 July 2001; accepted 11 August 2001

Our previous work has shown that PSD-95/SAP90 is required for NMDA receptor-mediated thermal hyperalgesia. To address the role of PSD-95/SAP90 in chronic pain, the present study investigated the effect of the deficiency of PSD-95/SAP90 on nerve injury-induced neuropathic pain. Following unilateral L5 spinal nerve injury, mechanical and thermal hyperalgesia developed within 3 days and persisted for 9 days or longer on the injured side. The intrathecal administration of antisense oligodeoxynucleotide specifically against PSD-95/SAP90, but not sense or missense oligodeoxynucleotide, dose-dependently delayed the onset of tactile allodynia and thermal hyperalgesia. These results suggest that PSD-95/SAP90 might be involved in the central mechanisms of the development of chronic neuropathic pain. *NeuroReport* 12:3251–3255 © 2001 Lippincott Williams & Wilkins.

Key words: Antisense technology; Hyperalgesia; Intrathecal injection; Neuropathic pain; PSD-95/SAP90; Spinal central sensitization; Spinal cord

INTRODUCTION

Chronic neuropathic pain is a physically and emotionally debilitating condition for which there is no adequate treatment. We have known that peripheral nerve lesions may generate a syndrome comprising, in addition to spontaneous pain, exaggerated responses to light touch (tactile allodynia) and heat stimuli (thermal hyperalgesia). However, the molecular mechanisms underlying these pathophysiological processes are unclear.

The NMDA receptor system has been implicated in the processing of spinal nociceptive information [1–6]. PSD-95/SAP90, a molecular scaffolding protein, has been identified to attach NMDA receptors to internal signaling molecules at neuronal synapses [7,8]. Therefore, PSD-95/SAP90 protein might be involved in many physiological and pathophysiological actions triggered via the activation of NMDA receptors in the CNS. This hypothesis has been supported by some data. For instance, suppression of PSD-95/SAP90 expression protected neurons against excitotoxicity produced by NMDA receptor activation [9]; enhanced NMDA-dependent long-term potentiation and impaired learning were observed in mice with mutant PSD-95/SAP90 protein [10]. Our previous work showed that PSD-95/SAP90 mRNA and protein were enriched in spinal cord and selectively distributed in the superficial dorsal horn, where PSD-95/SAP90 overlapped with NMDA receptors [11]. In addition, we found that PSD-95/SAP90 was required for NMDA receptor-mediated thermal hyperalgesia [11] and mediated the role of the NMDA receptor in determining the minimum alveolar anesthetic concentration of inhalational anesthetics [12]. However, the role of PSD-95/SAP90 in chronic persistent pain remains to be elucidated. In the present study, we investigated the effect of PSD-95/SAP90 deficiency on neuropathic pain after spinal nerve injury by using behavioral testing combined with antisense technology.

MATERIALS AND METHODS

Animal preparation: Male Sprague–Dawley rats (250–300 g) were housed in separate cages on a standard 12:12 h light:dark cycle, with water and food pellets available *ad lib*. All animal experiments were carried out with the approval of the Animal Care Committee at Johns Hopkins University and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The rats were implanted with an intrathecal catheter under halothane anesthesia. A polyethylene-10 catheter was inserted into the subarachnoid space at the rostral level of the spinal cord lumbar enlargement through an incision at the atlanto-occipital membrane according to the method described previously [13]. The animals were allowed to recover for a week before being used experimentally. Rats showing neurological deficits postoperatively were discarded from the study.

Experimental animal model for peripheral neuropathic pain: Peripheral neuropathic pain was induced as described previously [14] with minor modification. In brief, rats were anesthetized with halothane. The unilateral L6 transverse process was carefully removed to identify

visually the L4 and L5 spinal nerves. The unilateral L5 spinal nerve was isolated, tightly ligated with a 3-0 silk thread, and transected just distal to the ligature. A complete hemostasis was confirmed and the wound was sutured.

Intrathecal antisense oligodeoxynucleotide (ODN) administration: Antisense ODN was designed based on rat PSD-95/SAP90 mRNA and corresponded to the PSD-95/DLG/ZO-1 (PDZ) domain (nucleotides 241–258) of this mRNA [11,12]. Controls for the effect of antisense ODN included sense and missense (random ODN) ODNs. Sequences for the ODNs were as follows: antisense, 5'-TGTGATCTCCTCATACTC-3'; sense, 5'-GAGTATGAGGAGATCACA-3'; missense, 5'-AAGCCCTTGTTCCCATTT-3' [11,12]. All of the ODNs were searched to exclude nonspecificity of the sense or antisense ODNs and to show that missense ODN did not match any confounding sequences in the GenBank database (GenBank accession number: M96853). The ODNs were made and purified with the use of RP-HPLC (Integrated DNA Technologies, Inc., Coralville, IA). The ODNs were reconstituted in saline before administration. The rats were injected intrathecally with saline (10 µl), antisense ODN (12.5 µg, 25 µg or 50 µg/10 µl), sense ODN (50 µg/10 µl) and missense ODN (50 µg/10 µl), respectively, followed by an injection of 10 µl saline to flush the catheter, every 24 h for 4 days (from the day before L5 spinal nerve ligation until day 2 post-operation).

Behavioral testing: After surgery, the rats were returned to the animal room and maintained under the same conditions as the pre-operative period. A set of tests described below was conducted in all experimental animals 2 days prior to the surgery as baseline and on post-operative days 3, 5, 7 and 9.

Hindpaw withdrawal response to repeated mechanical stimuli: Mechanical stimuli were applied with two different calibrated von Frey filaments (8.01 mN and 120.21 mN). A single trial of stimuli consisted of eight applications of a von Frey filament within a 2–3 s period. Each trial was repeated 10 times at 3 min intervals on each hindpaw. The occurrence of hindpaw withdrawal in each of these 10 trials was expressed as a percent response frequency, and this percentage was used as an indication of the amount of hindpaw withdrawal.

Hindpaw withdrawal latency to noxious heat stimuli [15,16]: The rat was placed in a Plexiglas chamber on a glass plate under which a light box was located. A radiant heat stimulus was applied by aiming a beam of light through a hole in the light box to the heel of each hindpaw through the glass plate. The light beam was turned off when the rat lifted the foot, allowing the measurement of time between start of the light beam and the foot lift. This time was defined as the hindpaw withdrawal latency. Each trial was repeated five times at 5 min intervals for each side. A cut-off time of 20 s was used to avoid tissue damage to the hindpaw.

Statistical analysis: Data were expressed as mean ± s.e.m. and statistically analysed with one-way and two-way ANOVA followed by Student–Newman–Keul's t od. Statistical significance was set at $p < 0.05$.

RESULTS

PSD-95/SAP90 and mechanical pain hypersensitivity this study, we employed two kinds of von Frey filar to address the effect of PSD-95/SAP90 on mechanical hypersensitivity in the neuropathic pain model. was innocuous (8.01 mN), and another was no (120.21 mN). In the saline group ($n = 8$), following u eral L5 spinal nerve injury, mechanical pain hypersen ity developed within 3 days and persisted for 9 da longer in the ipsilateral hindpaw upon stimulation by of the von Frey filaments. Mean paw withdrawal resp to these mechanical stimuli displayed a significant inc on days 3, 5, 7 and 9 post-operation compared baseline ($p < 0.05$; Fig. 1). However, following the intr cal administration of the antisense ODN (50 µg/ $n = 8$), the pain hypersensitivity did not appear until post-operation (Fig. 1). Two-way ANOVA showed a s tically significant difference between the antisense g and saline group ($p < 0.05$). On post-operative day 9 antisense group showed mechanical hyperalgesia si to the saline group. However, sense (50 µg/10 µl; and missense (50 µg/10 µl; $n = 6$) ODNs had no effe the development of mechanical pain hypersensi ($p > 0.05$; Fig. 1).

PSD-95/SAP90 and thermal hyperalgesia: The ro PSD-95/SAP90 in the development of thermal hyperal was also investigated in the neuropathic pain model. I saline group ($n = 8$), we observed that thermal hype sia also developed within 3 days and persisted for 9 or longer in the ipsilateral hindpaw following unilater spinal nerve injury. Paw withdrawal latencies to stimuli exhibited a significant decrease on post-oper days 3, 5, 7 and 9 compared with baseline ($p < 0.05$; Fi The antisense ODN (50 µg/10 µl; $n = 8$) also delaye onset of thermal hyperalgesia, which did not appear day 7 post-operation following the application o antisense ODN (Fig. 2). On post-operative day 9 antisense group also showed thermal hyperalgesia si to the saline group. Meanwhile, sense (50 µg/10 µl; and missense (50 µg/10 µl; $n = 6$) ODNs had no effe the development of the thermal hyperalgesia ($p >$ Fig. 2).

Dose–response of the antisense ODN specifically ag PSD-95/SAP90: We tested three doses of this anti ODN (12.5 µg, 25 µg and 50 µg) and found that the eff this antisense ODN on hyperalgesia in this model was dependent. The intrathecal administration of 50 µg sense ODN ($n = 8$) delayed the development of hype sia until day 7 post-operation (Fig. 1, Fig. 2, Fi whereas 25 µg antisense ODN ($n = 6$) only delaye development of the hyperalgesia until day 5 post-oper (Fig. 3). In addition, we observed no effect of 1 antisense ODN ($n = 5$) on the development of s hyperalgesia (Fig. 3). No significant difference ex between the saline group and 12.5 µg antisense group.

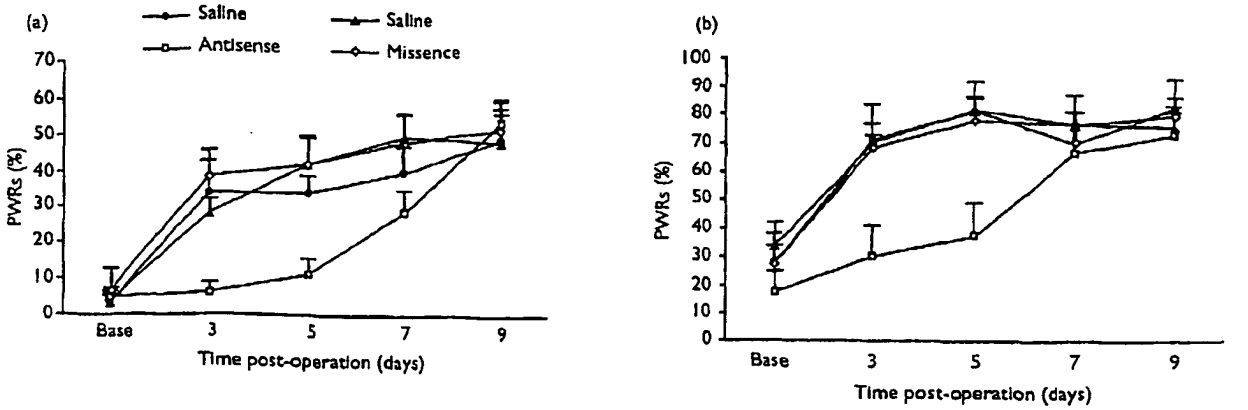

Fig. 1. Paw withdrawal responses (PWRs) to mechanical stimuli with von Frey filaments. (a) The innocuous (8.01 mN) von Frey filament; (b) The noxious (120.21 mN) von Frey filament. Values are mean ± s.e.m. Note that the intrathecal administration of the antisense ODN specifically against PSD-95/SAP90 can delay the onset of mechanical pain hypersensitivity in the neuropathic pain model, whereas sense and missense ODNs had no effect on this pain hypersensitivity.

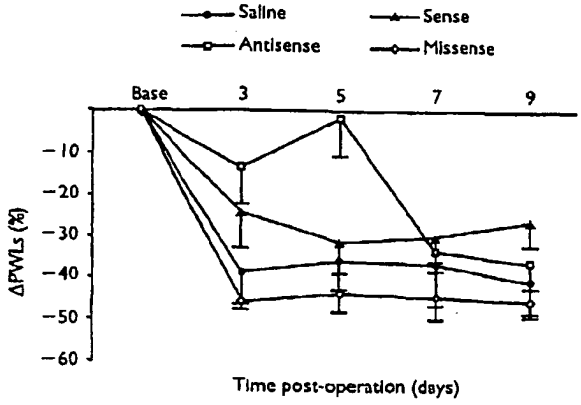

Fig. 2. Percent decrease of paw withdrawal latencies (ΔPWLs) to heat stimuli from baseline (Baseline was set as 0%). Values are mean ± s.e.m. Note that the intrathecal application of the antisense ODN specifically against PSD-95/SAP90 can delay the onset of the thermal hyperalgesia in the neuropathic pain model, while sense and missense ODNs had no effect on this thermal hyperalgesia.

DISCUSSION

Causalgia, a painful syndrome, is characterized by spontaneous burning pain combined with tactile allodynia and thermal hyperalgesia [17]. Although the disease has been known over a century, we have little knowledge about its pathogenesis, especially its molecular mechanisms. In the present study, we employed a neuropathic pain model via tight ligation and transection just distal to the ligature of the L5 spinal nerve to address this issue. This model manifests the symptoms of human patients with causalgia and can be used to answer questions regarding mechanisms underlying this syndrome.

In the past several years, progress in the development of molecular biology and the elucidation of gene sequences has created new approaches for studying biological functions and developing new diagnostic and therapeutic strategies. One of the most exciting advances has been the development of antisense technology, which represents a new strategy allowing modulation of protein synthesis with high specificity by preventing protein expression at the level of RNA or DNA. Many classical pharmacological approaches in neurobiological research are based on the inhibition of biologically active proteins, such as receptors for neurotransmitters, or enzymes involved in neurotransmitter synthesis or degradation. The application of antisense ODNs offers an alternative tool to manipulate selectively the expression of these active proteins in the central nervous system. This approach is especially useful when selective, high-affinity antagonists are not available. As a result, this technology has gained acceptance in the study of cell signaling mechanisms and the molecular basis of neuronal function [18,19]. In this study, using antisense ODN specifically against PSD-95/SAP90, we investigated the role of PSD-95/SAP90 in the development of chronic neuropathic pain. Our previous work has shown that the antisense ODN suppressed PSD-95/SAP90 expression in spinal cord, but it did not change the expression of NMDA receptor subunit NR2A/2B, neuronal nitric oxide synthase (nNOS) or SAP102 in spinal cord [11]. These results suggest that the antisense ODN we used is effective and specific. Our previous work also showed that the antisense ODN did not produce marked changes in either blood pressure or heart rate and did not influence the locomotor activity of experimental animals [12]. Therefore, the antisense ODN we designed can be safely used as a tool *in vivo*.

Consistent with a previous report, the present study showed that unilateral L5 spinal nerve injury induced the development of mechanical pain hypersensitivity and thermal hyperalgesia in the ipsilateral hindpaw within 3 days, and the spinal hyperalgesia persisted for 9 days or longer. Furthermore, we revealed for the first time that the PSD-95/SAP90 antisense ODN dose-dependently delayed the

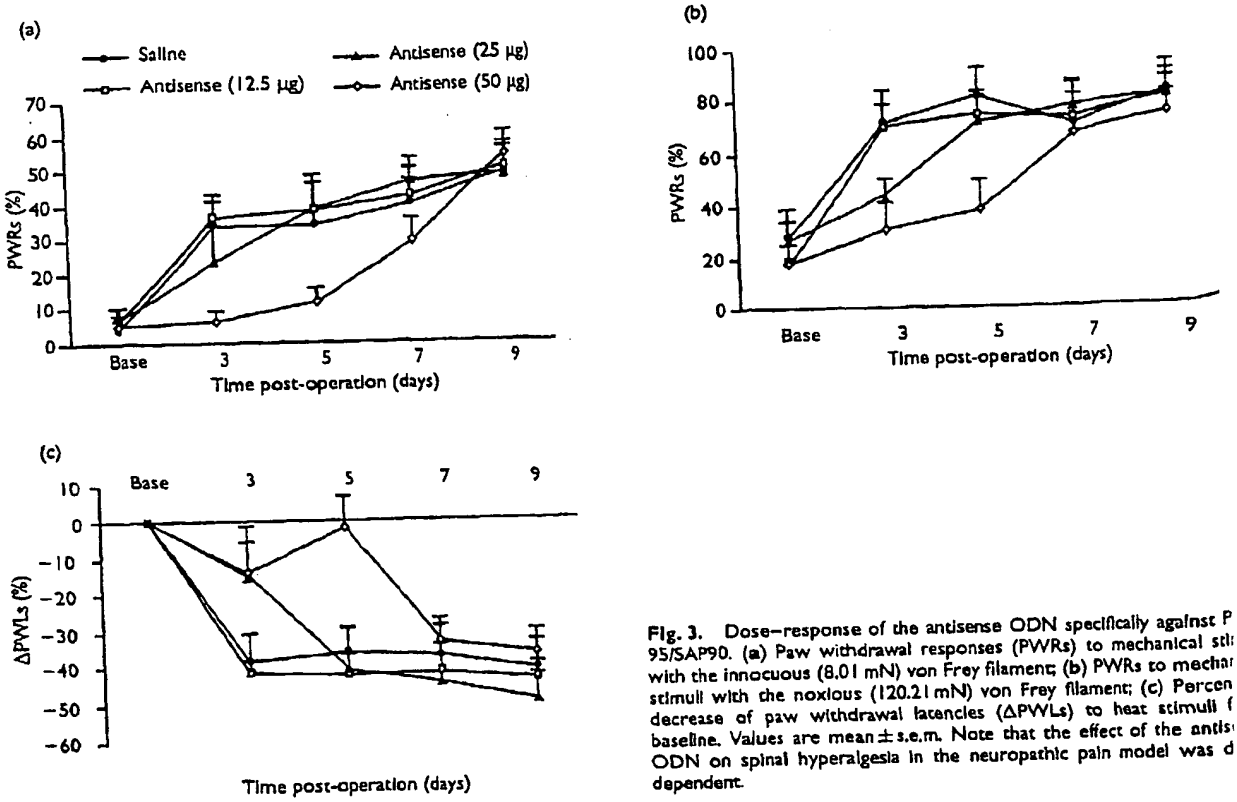

Fig. 3. Dose–response of the antisense ODN specifically against P 95/SAP90. (a) Paw withdrawal responses (PWRs) to mechanical sti with the innocuous (8.01 mN) von Frey filament; (b) PWRs to mechar stimuli with the noxious (120.21 mN) von Frey filament; (c) Percen decrease of paw withdrawal latencies (ΔPWLs) to heat stimuli f baseline. Values are mean ± s.e.m. Note that the effect of the antis ODN on spinal hyperalgesia in the neuropathic pain model was d dependent.

onset of the mechanical and thermal hyperalgesia in the chronic neuropathic pain model. Our findings suggest that PSD-95/SAP90 is involved in the pathophysiological mechanism of neuropathic pain and is necessary for the development of the hyperalgesia induced by peripheral nerve injury.

Several lines of evidence indicate that NMDA receptors play an important role in the pathogenesis of neuropathic pain following spinal nerve injury [20–24]. NMDA receptors are anchored in the postsynaptic density by interactions between the cytoplasmic C-terminal tails of their NR2 subunits and the PDZ domains of PSD-95/SAP90. The PDZ domains of PSD-95/SAP90 also bind to other postsynaptic membrane proteins, including potassium channels, tyrosine kinases and cell adhesion molecules. PSD-95/SAP90 functions as a scaffold to assemble a specific set of signaling proteins around the NMDA receptor. These proteins, such as nNOS, SynGAP and SPAR, may participate in downstream signaling by NMDA receptors [25]. Combined with the present work, we conclude that PSD-95/SAP90 may mediate the functions of NMDA receptors in chronic neuropathic pain. Although the detailed mechanism through which the deficiency of PSD-95/SAP90 affects peripheral nerve injury-induced spinal central sensitization remains obscure, the present study provides a novel insight into the mechanisms that underlie the chronic neuropathic pain state and a novel target for development of new pain therapies.

CONCLUSION

Unilateral L5 spinal nerve ligation produces persistent long-term mechanical and thermal hyperalgesia on injured side. We found for the first time that the deficie of PSD-95/SAP90 delayed the onset of mechanical thermal hyperalgesia in the chronic neuropathic model. Our results suggest that PSD-95/SAP90 might a key role in the mechanism of central sensitizatio chronic neuropathic pain.

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Acknowledgements: This study was supported by NIH grants RO1 GM49111 and RO1 HL39706. The authors would like to thank Mrs Claire F. Levine for technical assistance.